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Recombinant Toxins for Cancer Treatment

IRA PASTAN AND DAVID FITZGERALD

Recombinant toxins target cell surface receptors and antigens on tumor cells. They kill by mechanisms different from conventional chemotherapy, so that cross resistance to conventional chemotherapeutic agents should not be a problem. Furthermore, they are not mutagens and should not induce secondary malignancies or accelerate progression of benign malignancies. They can be mass-produced cheaply in bacteria as homogeneous proteins. Either growth factor–toxin fusions or antibody-toxin fusions can be chosen, depending on the cellular target.

RECOMBINANT TOXINS ARE HYBRID CYTOTOXIC PROTEINS made by recombinant DNA technology that are designed to selectively kill cancer cells. The cell-targeting moiety can be a growth factor or a single chain, antigen-binding protein. The toxic moiety is a portion of a bacterial or plant toxin. Immunotoxins are similar in concept but are composed of antibodies chemically linked to toxins.

More than 30 years ago chemotherapeutic drugs began to be used to treat cancer as a supplement to surgery and radiation therapy. Now that several decades have passed, it is clear that the current generation of chemotherapeutic drugs can achieve cures of certain leukemias and lymphomas and, in the adjuvant setting, prolong the lives of patients with breast cancer, ovarian cancer, and several other types of cancer. Because chemotherapy is not a cure for the common types of cancer in adults, new therapies must be developed.

One approach is to target a cytotoxic agent to the cancer cell (*1*). To accomplish this, the cytotoxic agent is attached to an antibody or a growth factor that preferentially binds to cancer cells. The targets for this type of therapy can be growth factor receptors, differentiation antigens, or other less characterized cell surface antigens. It is now established that many cancers overproduce growth factor receptors that can function as oncogenes and promote the growth of the cancer cells (*2–4*). For example, the epidermal growth factor receptor is present in large amounts (up to 3×10^6 receptors per cell) in many squamous cell and epidermoid carcinomas, glioblastomas, and some metastatic ovarian and bladder cancers (*5–7*). Normal cells contain as many as 3×10^5 receptors per cell (*8*). The interleukin-2 (IL-2) receptor is present in substantial numbers on the cells of patients with adult T cell leukemia (ATL; 3×10^4 receptors per cell) and in lower numbers in various other lymphoid malignancies (*9*).

Differentiation antigens that occur on normal cells such as B lymphocytes are often also present on tumor cells such as B cell lymphomas. Because such antigens are not present on the stem cells

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that produce B cells, any mature B cells that are killed by targeted therapy will be replaced from the stem cell population, whereas the cancer cells will not be replaced (*10*). Finally, there are antigens preferentially expressed on cancer cells whose functions are not yet understood. Some of these, such as carcinoembryonic antigens (*11*), are fetal antigens, which are either not present or only present in small amounts on normal adult tissues. This group also contains antigens of unknown origin that are only defined by their reactivity with a monoclonal antibody (*12–14*).

For targeted drug delivery to be successful, it is necessary that the cytotoxic agent be extremely active. Bacterial and plant toxins, which are some of the most cytotoxic substances known, act by irreversibly arresting the synthesis of protein in eukaryotic cells. *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT) do this by enzymatically inactivating elongation factor 2, an essential component of protein synthesis. Ricin and other plant toxins cleave a glycosidic bond in 28S ribosomal RNA (rRNA), thereby destroying the ability of ribosomes to synthesize protein. Because these toxins are catalysts with high turnover numbers, few molecules need to reach the cytoplasm to kill the target cell. These types of toxins enter cells by endocytosis and are processed to an active fragment that translocates across the cell membrane into the cytosol where the components of protein synthesis are located (Fig. 1). If one bypasses this pathway and directly injects an activated form of DT or PE into the cytosol, only the injection of a few molecules is necessary to kill the cell (*15*).

Initially, agents that delivered toxins to cancer cells were constructed by chemically coupling antibodies to toxins (*16–19*). Immunotoxins have been made by the coupling of antibodies to ricin A chain or to several other plant toxins and to modified forms of DT and PE. More recently, genetic engineering has been used to make recombinant toxins by fusing modified toxin genes to DNA elements encoding growth factors or the combining regions of antibodies (*20–23*). These chimeric genes are then expressed in *Escherichia coli*, from which the recombinant toxins are prepared.

Toxins in their native form require a minimum of three biochemical functions to kill cells: cell binding, cytotoxicity, and the ability to translocate the toxic activity into the cytosol. An advance in understanding the structural basis of each of these functional regions occurred with the crystallization of PE and the elucidation of its three-dimensional structure (*24*). PE is a single polypeptide chain with a size of 66 kD that is arranged into three major structural domains. This arrangement suggested that each domain could be responsible for one function. To test this hypothesis, Hwang *et al.* (*25*) isolated the PE gene from *Pseudomonas aeruginosa* and used a bacterial expression system to produce the whole toxin or fragments of the toxin corresponding to each structural domain (*25*). The results showed that domain Ia (amino acids 1 to 252) was the cell binding domain, domain II (amino acids 253 to 364) was required for translocation, and domain III (amino acids 400 to 613) was required for the adenosine diphosphate (ADP) ribosylation and inactivation of elongation factor 2. A subdomain termed Ib, which is composed of amino acids 365 to 399, has no known function, and most of it can be deleted without loss of activity (*26*).

The steps involved in the killing of cells by PE are shown in Fig. 1. DT probably follows a similar pathway. Soon after toxin binding, the toxin–receptor complex is internalized by the pathway of receptor-mediated endocytosis (*27, 28*). The toxin travels via clathrin-coated pits into endocytic vesicles where the toxin is cleaved into two pieces by a combination of a proteolytic step and reduction of a disulfide bond (*29*). The 37-kD fragment derived from the COOH portion, which contains part of domain II and all of domain III is translocated to the cytosol where it arrests protein synthesis and causes cell death (*29*). Although Fig. 1 illustrates translocation occurring from an endocytic vesicle, recent data suggest translocation may occur in the endoplasmic reticulum.

Diphtheria toxin is also a single chain toxin in which the functional domains are arranged in the opposite order from PE, with the ADP ribosylating function at the NH_2-terminus and the binding domain at the COOH-terminus (*30, 31*). DT made in *E. coli* is thought to be proteolytically cleaved after binding to target cells (*32*). Ricin is composed of two subunits linked together by a disulfide bond. The A chain contains the enzymatic activity; the B chain binds to galactose residues present on many different cell surface glycoproteins and glycolipids. *Pseudomonas* exotoxin, diphtheria toxin, and ricin are each synthesized as a single polypeptide chain. The chain is later proteolytically cleaved into two fragments, and the fragment containing the toxic enzymatic activity is translo-

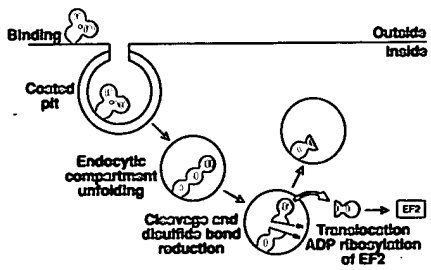

Fig. 1. Journey to the cytosol. Domain I of PE binds to a surface receptor and is taken into the endocytic compartment via coated pits. The low pH of the endosome and possibly other factors cause the toxin to unfold. Toxin is then proteolytically cleaved within domain II. Cleavage is followed by reduction and the release of a 37-kD COOH-terminal fragment that translocates to the cytosol, and there ADP ribosylates elongation factor 2.

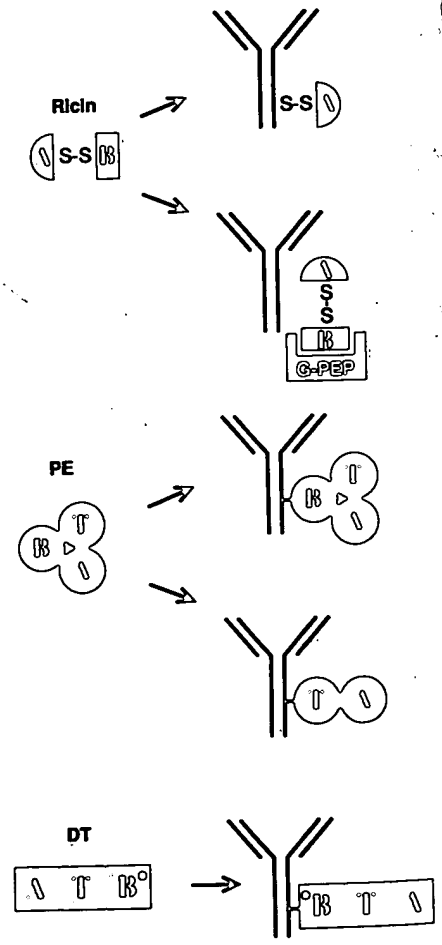

Fig. 2. Strategies for making immunotoxins. Ricin A chain is chemically linked to a monoclonal antibody or the B chain of whole ricin is blocked with a glycopeptide and modified ricin linked to an antibody. Whole PE is conjugated to an antibody via domain I or domain I is deleted and then PE40 is conjugated to the antibody. A mutant form of DT with reduced binding activity is attached to an antibody molecule.

Table 1. Clinical trials with immunotoxins. About 500 individuals have been treated in clinical trials. All but one of these trials used toxins chemically linked to antibodies (immunotoxins). Administration of the recombinant toxin DAB 486–IL-2 has begun in individuals with ATL and other IL-2 receptor–positive malignancies. The abbreviations used are as follows: RTA, ricin A chain; dgRTA, deglycosylated ricin A chain; CLL, chronic lymphocytic leukemia; T-ALL, T-cell acute lymphocytic leukemia.

Immunotoxin	Disease	Phase	References
Xomazyme-Mel–RTA	Melanoma	1/2	(*83*)
791T/36-RTA	Colorectal cancer	1	(*56*)
260 F9-RTA	Breast cancer	1	(*54, 55*)
Anti-B4–B-ricin	B cell malignancies	1/2	(*37*, 84**)
T-101–RTA	CLL/T-ALL	1	(*18, 85*)
Anti-CD22 RTA dgRTA	B cell malignancies	1	(*53*)
Anti-CD22 Fab dgRTA	B cell malignancies	1	(*53*)
Anti-Tac(Fv)–PE40	ATL	1	(*86**)
OVB3-PE	Ovarian cancer	1	(*57*)
DAB 486–IL-2	ATL and other lymphoid tumors	1	(*21**)

*Results of clinical trials are unpublished.

cated into the cytosol (*29, 32*). PE is not cleaved (processed) until it enters the target cell where both proteolytic cleavage and disulfide bond reduction occur. In contrast, diphtheria toxin is clipped shortly after secretion, and ricin is cleaved within the seed where it is synthesized (*33*). In both examples the two fragments remain held together by a disulfide bond which is ultimately broken within the target cell (*34*).

Immunotoxins

To construct active immunotoxins, the toxin must be modified so that its interactions with cellular receptors are diminished or abolished (Fig. 2). As a consequence, toxin entry is mediated by antibody binding. With ricin, this is accomplished through removal of the B chain (*35*), blockage of the galactose binding site (*36*) or attachment of a galactose-rich carbohydrate to the B chain (*37*). With PE, this modification occurs when the antibody is coupled to domain I of PE, which interferes with the binding of domain I to the PE receptor (*38*), or when domain I (amino acids 1 to 252) is genetically deleted and coupled to antibody to domain II (*39*). With DT, this modification occurs through the mutation of a key amino acid in the binding domain near the COOH-terminus or through the removal of a portion of the COOH-terminus that is responsible for binding to cells (*40, 41*).

Ricin is purified from castor beans and has been available for immunotoxin production for many years. More recently, a recombinant form of the A chain has been produced in *E. coli* (*42*). PE has been obtained from the culture medium of *P. aeruginosa*, but both the whole toxin and mutant forms have been produced in *E. coli* (*20*) as have been mutant forms of DT.

The activity of an immunotoxin is initially assessed by measuring its ability to kill cells with target antigens on their surfaces. Because toxins act within the cell, receptors and other surface proteins that naturally enter cells by endocytosis usually make good targets for immunotoxins, but surface proteins that are fixed on the cell surface do not. However, if several antibodies recognizing different epitopes on the same cell surface protein are available, it is useful to test them all, because some, perhaps by producing a conformational change in the target protein's structure, may induce its internalization or direct its intracellular routing to an appropriate location for toxin translocation (*43, 44*). Also, it is possible to induce internalization of a target surface protein if the immunotoxin contains a form of PE or ricin in which the binding of the toxin moiety to its receptor, although weakened by chemical modification, still occurs and promotes internalization since toxin receptors are efficiently internalized (*14, 37, 41*).

Many immunotoxins produce selective killing in cell culture, but only a few of these have been able to cause substantial or complete tumor regression in animals. Ricin A chain coupled to antibodies recognizing B cell specific antigens have caused complete regression of B cell lymphomas in mice (*45*). When antibodies to carcinomas are used, only partial responses have been observed (*46–48*). Regression of human carcinomas growing in immunodeficient mice has been achieved by treatment with monoclonal antibodies reacting with ovarian, colon, and breast cancers coupled either to PE itself or to PE40, a mutant form of PE in which the cell binding domain was deleted (*14, 49, 50*). One of these, B3-PE40, causes complete regression of human tumors growing in mice (*51*).

Several immunotoxins have been developed and approved for human trials. Two different kinds of trials have been conducted. The first involves the ex vivo addition of immunotoxins to harvested bone marrow to eliminate contaminating tumor cells before reinfusion in patients undergoing autologous bone marrow transplantation. A variety of antibodies, linked to ricin or ricin A chain, including anti-CD5 and anti-CD7, have been used for this purpose (*52*). The second kind of trial involves the parenteral administration of immunotoxins, either regionally (such as the peritoneal cavity) or systemically to patients with cancer. These have been primarily Phase 1 and 2 trials in patients in which conventional treatments have failed, and the patients have a large tumor burden. A list of clinical trials, either completed or still in progress, is provided in Table 1. Definite responses have been noted with lymphomas, and these trials will be expanded (*53*). So far, the antibodies used for the preparation of immunotoxins to treat carcinomas or other solid tumors have been found to react with important normal human tissues (such as neural tissue and bone marrow) and produce dose-limiting toxicity without significant clinical responses (*54–57*).

Genetically Engineered Recombinant Toxins

The production of immunotoxins by chemical coupling methods is expensive because it requires large amounts of antibody and toxin. Furthermore, the chemical conjugation methods used produce heterogeneous products, and antigen binding is often affected by chemical derivatization. It has been possible to overcome these difficulties and to create cytotoxic agents by genetic engineering. Both PE and DT have been used to make recombinant toxins in *E. coli* (*20, 23*). Ricin-based molecules have been difficult to produce probably because the A chain of the plant toxin must be attached to the cell recognition domain by a disulfide bond, and disulfide-linked subunits are difficult to produce in bacteria. The addition of a proteolytic cleavage sequence may help to overcome this difficulty (*58*).

The x-ray crystallographic structure of PE has been used as a guide for the synthesis of genetically engineered recombinant toxins (Fig. 3). The specific binding of PE to target cells occurs through an interaction of domain I with cellular PE receptors (*59, 60*). The junction of domain I with domain II occurs between Glu^{252} and Gly^{253}. Therefore, in constructing recombinant toxins, domain I was deleted, and the COOH-terminal–amino acid of various growth factors and other targeting molecules were fused directly to Gly^{253} of PE (occasionally a few additional amino acids have been added as a link between the COOH-terminus of the growth factor and Gly^{253} of PE to make cloning more convenient). One widely studied molecule is TGF-α–PE40, which was constructed by replacing domain I of PE with transforming

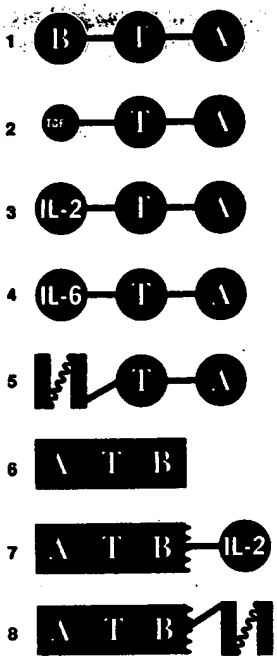

Fig. 3. Strategies for making recombinant toxins. The DNA coding for domain I of PE is removed and replaced with cDNAs for ligands such as TGF-α, IL-2, IL-6, and single chain antibodies. The cDNAs are placed at the 5′ end of each construction and preserve the relative position of the binding function to the other functional domains of PE. Binding by DT is mediated by sequences at the 3′ of its structural gene. Therefore, ligands are added to a truncated form of DT at the 3′ end.

growth factor alpha (TGF-α). In this chimeric toxin, the 23-kD domain I is replaced by the 6-kD growth factor (Fig. 3), to produce a chimeric toxin that selectively binds to and kills cells with epidermal growth factor (EGF) receptors. The structure of the plasmid encoding TGF-α–PE40 is shown in Fig. 4 with the cDNA encoding TGF-α inserted adjacent to the PE40 gene. The expression vector used for the production of TGF-α–PE40 and other PE-based chimeric toxins in *E. coli* contains the bacteriophage T7 promoter, an efficient ribosome binding site and an Nde I site (CATATG), which encodes a methionine initiation codon where targeting ligands can be conveniently inserted. The gene encoding the phage T7 polymerase is inserted into the *E. coli* chromosome next to a *lac* promoter so that it can be induced by the addition of isopropylthiogalactoside (IPTG) (*61*).

TGF-α–PE40, like other chimeric toxins made in *E. coli*, accumulates in large amounts within the cell in insoluble aggregates (inclusion bodies). After cell disruption, inclusion bodies are easily isolated and can contain up to 90% recombinant protein in an insoluble form. The protein is then dissolved in a strong denaturant such as 7 M guanidine-HCl, renatured, and can be

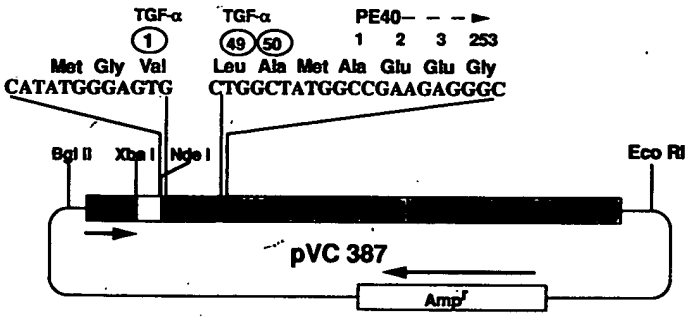

Fig. 4. Expression of recombinant toxins from plasmids containing the T7 promoter. Production of PE recombinant toxins is driven by T7 polymerase, which is made by induction of the lactose operon situated on the chromosome of *E. coli* BL21 (λDE3). The polymerase acts on the T7 promoter immediately upstream of the structural gene for the recombinant toxin. TGF-α is shown fused with PE40. This construction is inserted into an Nde I site, which provides the first methionine codon to begin the translation of the fusion protein. Additional amino acids are present at the junction of two structural genes (*79*).

Table 2. Targets for recombinant toxins.

Surface target	Relevant cancers	Recombinant toxin
EGF receptor	Lung, head and neck, bladder, glioblastoma, breast	TGF-α–PE40 (*88*)
IL-2 receptor	ATL	IL-2–PE40 (*64*)
	T cell lymphomas	DT–IL-2 (*23*)
		Anti-Tac(Fv)–PE40 (*22*)
		DT–anti-Tac(Fv) (*87*)
IL-6 receptor	Myeloma, hepatoma	IL-6–PE40 (*62*)
		IL-6–PE66^{Glu} (*89*)
Erb-B2 protein	Breast, ovarian, bladder	Anti-ErbB2(Fv)–PE40
Cancer-associated antigens	Carcinomas	B3(Fv)–PE40 (*80*)

purified to near homogeneity in two or three steps by conventional column chromatographic methods (*62*). TGF-α–PE40 binds to EGF receptor-containing cells with about the same affinity as TGF-α, and its toxicity on these cells is directly related to the number of receptors present.

Recombinant Toxins and Their Targets

The EGF receptor (EGF-R) has been the subject of intense study and shown to act as an oncoprotein when it is overexpressed in normal cells (*2*). TGF-α–PE40 is extremely cytotoxic to cells that contain EGF receptors and has been shown to have an antitumor effect in animals bearing tumors that have more EGF receptors than normal cells (*50, 63*). Clinical trials have just begun in which TGF-α–PE40 is instilled into the urinary bladder to treat superficial bladder cancer. Recombinant toxins have now been made by combining IL-2, IL-4, IL-6, IGF-1, and acidic fibroblast growth factors (FGF) with PE40 (*62, 64–67*) and DT with IL-2 and melanocyte-stimulating hormone (*21, 68*) (Fig. 3). Each was cytotoxic to cell lines containing the appropriate receptors. Furthermore, anti-tumor activity in animals bearing human cancers has been demonstrated with TGF-α–PE40, IL-2–PE40, and IL-6–PE40 (*69, 70*). Another way to assess efficacy of such agents is to test them against cancer cells directly isolated from patients. DT–IL-2 has activity against fresh leukemic cells from patients with ATL (*71*) and is now being evaluated in Phase 1 trials in individuals with various lymphoid malignancies. Table 2 summarizes some tumor types that contain large numbers of receptors and may serve as targets for recombinant toxin therapy.

Although growth factors fused to toxins have proved to be effective cytotoxic agents, a concern has been raised that some of these might promote tumor growth in certain circumstances (for example, if less than a full toxic dose were administered) (*65–72, 73*). Because antibodies to growth factor receptors do not usually have agonist effects, the antigen combining region of these antibodies can be used for targeting. This approach takes advantage of the finding that the variable regions of the light and heavy chains of antibodies can be combined into a single chain form that retains high affinity binding to antigen (*74–76*). Accordingly, a complementary DNA (cDNA) from the antibody to Tac (anti-Tac) was used to construct an Fv fragment that was fused to PE40 (*22*) (Fig. 3). The Fv portion of an antibody molecule is composed of two variable regions of the light and heavy chains and these can be held together by a linking peptide to make a single chain Fv. This single-chain recombinant toxin anti–Tac(Fv)–PE40, which is under preclinical development has potent cell-killing activity against cells with IL-2 receptors and against cells directly isolated

from ATL patients (77). The approach, of combining single chain antibodies with PE40, has been extended to several other antibodies, including an antibody to the human transferrin receptor (78), OVB3 (79), and B3 (80). The antibody to B3, which binds to a carbohydrate antigen expressed on the surface of many carcinomas (81), has been used to make a single-chain recombinant toxin that causes the complete regression of human tumors in mice (80).

Problems and Prospects

Toxins are foreign proteins and highly immunogenic. Therefore, in the absence of immunosuppression, neutralizing antibodies develop about 10 days after exposure to toxin (57, 82) and antibodies to DT already exist in most individuals who have received immunizations with diphtheria, pertussis, and tetanus (DPT). Animal studies have shown that immunotoxins and recombinant toxins act quickly so that tumors regress in a few days. Similar rapid responses should occur in humans. Nevertheless for long-term therapy, concomitant administration of immunosuppressive agents will be necessary to prolong the treatment period or the same antibody or ligand can be used to target different toxins. Many of the patients being treated in the current clinical trials have received extensive chemotherapy and are severely immunosuppressed so that the treatment period can be extended. In this review, we have discussed the utility of three toxins that are under clinical development, but there are still concerns associated with their use. Many people in the Western world have neutralizing antibodies to DT as a result of childhood immunization, and this has caused concern about the utility of recombinant toxins containing DT. Ricin A chain and blocked ricin both make active immunotoxins, but the recombinant molecules produced so far have low cytotoxic activity. The advantage of PE is that its structure is known. And, by means of this as a guide, the cell binding domain has been successfully replaced with more than a dozen different ligands including several different single chain antibodies. Furthermore, less than 3 percent of humans have pre-existing antibodies to PE. In the next several years, we anticipate that the major role of immunotoxins and recombinant toxins will be in the adjuvant setting for the treatment of metastatic disease that remains after surgery or radiation therapy.

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